

In Vivo Resistance of a Laboratory-Selected *Aspergillus fumigatus* Isolate to Amphotericin B

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The fungal burdens (number of CFU per pair of lungs) in mice infected with *Aspergillus fumigatus* AB16.4 (for which the amphotericin B [AMB] MIC was elevated) and W73355 (drug-susceptible parent) were reduced by 21 and 81%, respectively, after 5 days of AMB treatment (2 mg/kg/day), indicating that AB16.4 also shows reduced susceptibility to AMB in a murine pulmonary aspergillosis model.

Correlation of (i) reduced in vitro susceptibility of *Aspergillus* species to antifungal drugs with (ii) diminished response to therapy in an animal model will be useful for predicting the outcome of antifungal therapy against *Aspergillus* infection. Previous investigations of the in vitro-in vivo correlation of amphotericin B (AMB) resistance in *Aspergillus fumigatus* resulted in conflicting data. Odds et al. (6) and Johnson et al. (1) have found a poor in vitro-in vivo correlation for AMB resistance in *A. fumigatus* with a murine model for disseminated aspergillosis. Also, Verweij et al. (7) reported that an *A. fumigatus* isolate refractory to AMB therapy in an invasive murine aspergillosis model consistently provided AMB MICs considered to be in the susceptible range. On the other hand, Lass-Flörl et al. (2) reported a correlation between AMB MICs and the clinical outcome of AMB therapy in patients with *Aspergillus* infection. The higher the AMB MICs were, the poorer the outcome of drug therapy was.

We previously selected in the laboratory six *A. fumigatus* isolates (designated AB16.1 to AB16.6) showing reduced in vitro susceptibility to AMB from the drug-susceptible clinical isolate W73355 (3). To study a possible in vitro-in vivo correlation of AMB resistance in *Aspergillus*, we examined the in vivo susceptibility of *A. fumigatus* AB16.4 by a murine pulmonary aspergillosis model and compared the results with those obtained with parent strain W73355.

The MICs of voriconazole (Pfizer Pharmaceuticals, New York, N.Y.), itraconazole (Janssen Pharmaceutica, Beerse, Belgium), posaconazole (Schering-Plough Research Institute, Kenilworth, N.J.), ravuconazole (Bristol-Myers Squibb Institute for Medical Research, Princeton, N.J.), and AMB (Sigma Chemical Company, St. Louis, Mo.) for *A. fumigatus* W73355 and AB16.4 were determined in RPMI 1640 medium by the M38-A broth microdilution method (5), except that the MIC was defined as the concentration of the drug that produced no visible growth. Drug concentrations ranging from 0.015 to 16 µg/ml were used for MIC determinations. Where applicable, comparable concentrations of the solvent dimethyl sulfoxide were used as a control. Each MIC determination was repeated

at least once, and the results were the same or ± 1 twofold dilution.

The effect of AMB on the growth of *A. fumigatus* W73355 and AB16.4 in liquid medium was determined as follows. Cultures were grown in 100 ml of peptone yeast extract glucose broth from conidia (10^4 /ml) in the presence or absence of AMB (0.25 to 8 µg/ml) at 35°C for 48 h with gentle agitation on a gyratory shaker (150 rpm). The mycelia were collected on Whatman no. 2 filter paper and washed by vacuum filtration; the wet weight of mycelia was determined and plotted against the concentration of the drug to determine the effect of AMB on growth. The experiment was repeated once, and similar results were obtained in both experiments.

The effect of AMB on the growth of *A. fumigatus* W73355 and AB16.4 on solid medium was studied as follows. Briefly, replicate Sabouraud dextrose agar plates with and without AMB (0.25 to 16 µg/ml) were inoculated with 0.005 ml of a conidial suspension (10^6 conidia/ml) at the center; the fungi were grown for 5 days at 35°C, and the diameter (millimeters) of the fungal colony in each plate was determined. The mean colony diameter was plotted against the drug concentration to determine the effect of AMB on growth. The experiment was repeated once, and almost identical results were obtained in both experiments.

To study the in vivo susceptibility of *A. fumigatus* W73355 and AB16.4 to AMB, 6-week old female DBA/2J mice (Jackson Laboratories, Bar Harbor, Maine) were immunosuppressed by subcutaneous cortisone acetate injections (250 mg/kg/dose) on days –3, –1, and 1, where day 0 is the day of infection, and infected with 10^6 conidia (0.05 ml) delivered to the nares with a micropipette. Intraperitoneal AMB treatment (2 mg/kg/day) was initiated on day 1 and continued for 5 days. Animals were observed at least twice each day, and deaths were recorded. The effectiveness of AMB treatment was assessed by determining the fungal burdens (4) in the lungs of animals deceased and sacrificed on days 1 to 5. All animal experiments were performed in accordance with the guidelines of the Animal Investigation Committee of Wayne State University, Detroit, Mich.

The concentrations of triazoles that produced no visible growth of W73355 and AB16.4 were identical (range, 0.062 to 0.5 µg/ml), whereas those of AMB were 0.5 and 8 µg/ml, respectively. As shown in Fig. 1, AMB inhibited the growth of

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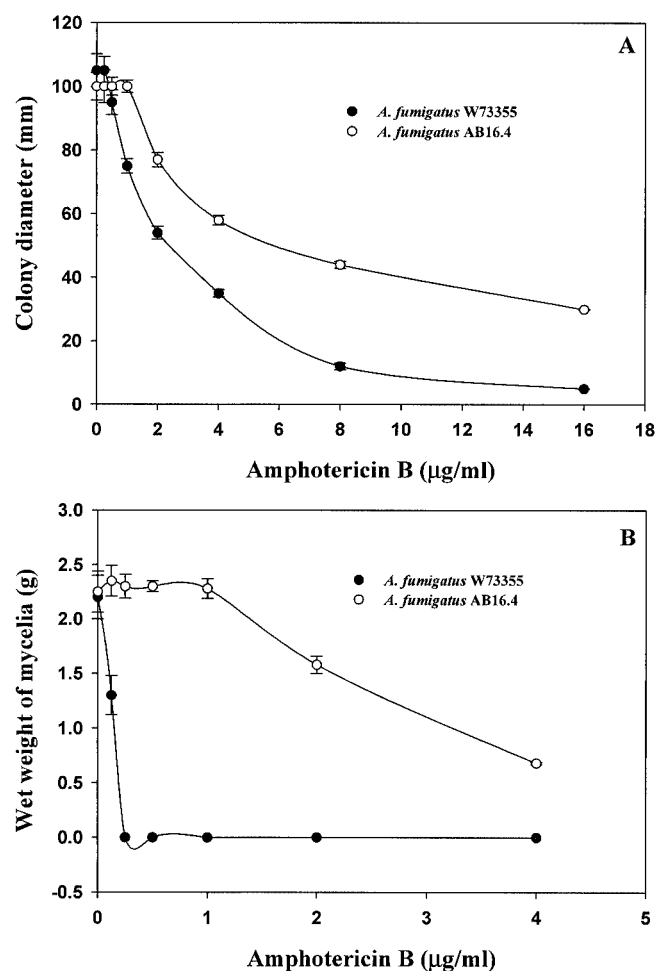


FIG. 1. Comparison of the inhibitory effects of various concentrations of AMB on *A. fumigatus* W73355 and AB16.4 on Sabouraud dextrose agar (A) and peptone yeast extract glucose broth (B). Each point represents the mean of three independent experiments \pm the standard deviation.

AB16.4 on solid and liquid growth media in a concentration-dependent manner. No inhibition of growth was obtained in the presence of AMB up to 1 µg/ml for AB16.4, and approximately 60% inhibition was obtained in the presence of 16 µg/ml. On the other hand, AMB at 1 and 16 µg/ml inhibited the growth of W73355 on Sabouraud dextrose agar by 20 and 100%, respectively. Similarly, the growth of AB16.4 in peptone yeast extract glucose broth was unaffected by AMB up to 1 µg/ml; approximately 30 and 66% inhibition was obtained at 2 and 4 µg/ml, respectively, compared to the growth of a drug-free control; and complete inhibition of growth was obtained at 8 to 16 mg/ml, whereas AMB at 0.25 µg/ml completely inhibited the growth of W73355. These results show that an almost 32- to 64-fold higher concentration of the drug is required to obtain the same amount of growth inhibition by AMB for AB16.4 as for parent strain W73355.

As shown in Fig. 2, the fungal burden in the lungs of animals ($n = 24$) infected with W73355 but untreated with AMB was $30,862 \pm 17,092$ CFU per pair of lungs whereas that in animals treated with AMB (2 mg/kg/day) for 5 days was only $5,969 \pm$

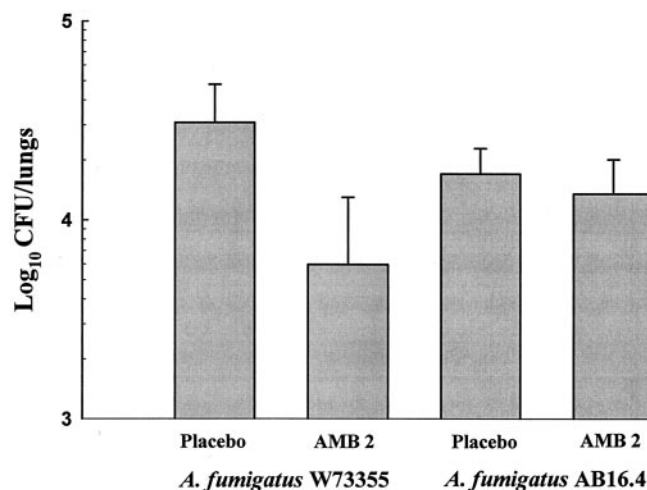


FIG. 2. Comparison of the cumulative fungal burdens (number of CFU per pair of lungs) in the lungs of animals infected with *A. fumigatus* W73355 and AB16.4 after 5 days of treatment with AMB. Each histogram represents the mean number of CFU per pair of lungs obtained for 24 animals, and the vertical bar on each histogram denotes the standard deviation. AMB 2, AMB at 2 mg/kg/day.

7,029 CFU per pair of lungs (~80% reduction compared to placebo; $P = 0.057$). In contrast, the fungal burden in the lungs of animals ($n = 24$) infected with AB16.4 but untreated with the drug was $17,025 \pm 5,866$ CFU per pair of lungs whereas that of animals that received AMB treatment (2 mg/kg/day) for 5 days was $13,514 \pm 6,682$ CFU per pair of lungs (~20% reduction compared to the placebo; $P = 0.588$). These results show that AMB treatment at a dose of 2 mg/kg/day for 5 days is highly effective at reducing the fungal burden of animals infected with drug-susceptible parent strain W73355 but not that of the animals infected with isolate AB16.4, which has reduced in vitro susceptibility to AMB.

This study was designed to measure the effectiveness of short-term (5 days) AMB treatment in decreasing the fungal burden in the lungs of animals infected with *A. fumigatus* isolates for which the MICs of AMB were low and high. Hence, we did not use the survival of the animals to evaluate the efficacy of AMB treatment. We predicted that if the isolate for which the MIC was high were less susceptible to AMB treatment in vivo, then the cumulative fungal burden in the lungs of animals infected with AB16.4 would be relatively higher than that in the lungs of animals infected with W73355. These results show that reduced in vitro susceptibility of *A. fumigatus* AB16.4 to AMB is correlated with its diminished susceptibility to AMB therapy in a murine pulmonary aspergillosis model.

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